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(57) Abstract

This invention provides a chimeric protein comprising an amino portion having the amino acid sequence of interleukin-3 and a carboxy portion having the amino acid sequence of mutein interleukin-6.

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Chimeric Interleukin-3/Mutein Interleukin-6 Lymphokine

The present invention relates to a chimeric protein comprised of Interleukin-3 and a mutein of Interleukin-6. The mutein of Interleukin-6 (mIL-6) has the first two cysteine residues replaced with any other amino acid residue. The chimera may be constructed according to the following formula:

IL-3--L--mIL-6

in which IL-3 represents Interleukin-3, mIL-6 represents the mutein of Interleukin-6 and L represents the first twenty-two amino acid residues of the Interleukin-6 mutein. (See Figure 2) An example of the nucleic and amino acid sequence of the chimeric IL-3/mIL-6 protein of the present invention is shown below in SEQ. ID. NO. 1. The invention also includes nucleic acid sequences encoding such proteins, plasmids and vectors containing such nucleic acid sequences, cells capable of expressing the protein and methods of using the protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the three nucleic acid fragments used to construct the chimeric IL-3/mIL-6 protein of the present invention. Line A represents nucleic acid sequences encoding human IL-3. Line B represents the restriction fragment obtained from the IL-3 sequence represented by line A by endonuclease digestion with *Nco*I and *Dde*I. The plasmid containing the fragment is designated p570. Lines C and C' show the 3' end of the IL-3

restriction fragment (line B), which lacks the nucleic acid sequence that encodes the eleven amino acids from the carboxy terminal end of native IL-3 (line A). Lines D and D' represent an oligonucleotide pair that contains the nucleic acid sequences for the last eleven amino acids of IL-3 and the first four amino acids of mIL-6, all of which are forfeited during digestion of the nucleic acid sequences encoding IL-3 with *Nco*I and *Dde*I endonucleases and mIL-6 with *EcoR*II and *Hind*III endonucleases. Line E represents the *EcoR*II/*Hind*III restriction fragment encoding mIL-6 which lacks the first four amino acid residues from the amino terminal end of the molecule. Line E' represents the portion of the pKK223-2 IL-6 SSCC plasmid which contains the nucleic acid sequences that encode the mIL-6 protein which lacks the first four amino acid residues from the amino terminal end of the molecule. Line E" represents the sequence from which the *EcoR*II/*Hind*III restriction fragment (line E) is obtained.

Figure 2 illustrates the relative positions of the IL-3, L and mIL-6 portions of one embodiment of the chimera of the present invention.

Figure 3 illustrates the expression vector pSE420. The pSE420 vector contains the $lacI^q$ gene, which allows for regulated expression in *E.coli* HB101. Transcriptional control is via the *trc* promoter and utilizes the highly efficient translation re-initiation characteristic of mini-cistron systems. The incorporation of upstream anti-termination and g10 ribosome binding sequences ensures high level translation of inserts cloned into its polylinker. Digestion of pSE420 with *Nco*I and *Kpn*I allows subsequent mobilization of the IL-3/mIL-6 chimera, by *Nco*I/*Kpn*I digestion of the IL-3/mIL-6-pKK233-2 plasmid, into this protein expression system.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In this specification, Interleukin-3 (IL-3) and Interleukin-6 (IL-6) refer to human IL-3 and human IL-6, respectively. The terms IL-3 and IL-6 include proteins described in the literature as having the same name as IL-3 or IL-6. For example, IL-3 is also known as multi-colony-stimulating factor (multi-CSF). IL-6 is also known as interferon-ß-2 (IFN-ß-2), B-cell stimulation factor-2 (BSF-2), B-cell hybridoma/plasmacytoma growth factor (HPGF or HGF), 26 kDa protein and hepatocyte stimulating factor (HSF).

The DNA and amino acid sequences of IL-3 are published and may be constructed by methods known in the art; see, for example, PCT publication WO 88/00598, published 28 January 1998 and PCT publication WO92/04455, published 19 March 1992.

The amino acid sequence of IL-6 has been described in the literature; see, for example, Figure 2A of Brakenhoff et al., Journal of Immunology 139, 4116-4121 (1987) and Figure 1 of Clark et al., PCT publication WO 88/00206, published 14 January 1988. These references also contain the cDNA sequence that corresponds to native IL-6 mRNA.

A mutein of IL-6 in which the first two cysteine residues are replaced by other amino acids has been described by Skelly et al., in co-pending U.S. patent application 07/907,710, which is incorporated herein by reference. mIL-6 has also been described in the literature; see for example, Dagan et al., *Protein Expression and Purification* 3, 290-294 (1992) and Snouwaert, J., et al., *J. Immunol.* 146, 585-591 (1991). These references define native IL-6 as a protein having 185 amino acids starting with alanine at amino acid position one.

mIL-6 is a mutein wherein the cysteine residues corresponding to amino acid positions 45 and 51 of native IL-6 have been replaced by other amino acids, while the cysteine residues corresponding to amino acid positions 74 and 84 have been retained. Preferably, the cysteine residues are replaced by neutral amino acids such as serine or alanine.

DNA sequences that encode native IL-3 and IL-6 include, but are not limited to, mammalian sources such as murine, pan and human sequences.

The term "chimera" or "chimeric protein" in this specification is understood to refer to a non-naturally occurring protein that is formed by joining one genetically distinct protein to another genetically distinct protein, end to end, in such a way that the biological activity of both proteins is retained or enhanced.

The term "fusion protein" in this specification is understood to refer to a protein that is produced in a system in which the desired protein is linked to a fusion partner, usually for the purpose of expediting expression or purification. Some suitable fusion partners include *trpE*, b-galactosidase, Protein A, maltose binding protein, etc. Once the fusion protein is produced, the desired protein may be cleaved from the fusion partner.

The words "amino acid" in this specification are understood to mean the approximately 21 naturally occurring a-amino acids or their analogs.

Preparation

The chimeric IL-3/mIL-6 protein and fragments thereof may be prepared by methods known in the art. A preferred method of preparing the chimeric protein of the present invention involves isolating DNA sequences that encode IL-3 and mIL-6, joining the IL-3 and mIL-6 encoding sequences in frame to form a single nucleic acid sequence that encodes the IL-3/mIL-6 chimera; amplifying or cloning the DNA in a suitable host; expressing the DNA in a suitable host; and harvesting the protein.

More specifically, a chimeric IL-3/mIL-6 nucleic acid sequence may be constructed as follows:

1) the major portions of the IL-3 and mIL-6 genes are excised with restriction endonucleases from plasmids containing the genes;

2) an oligonucleotide is used to replace sequences from IL-3 and mIL-6 which are lost as a result of the excision of the IL-3 and mIL-6 portions of the genes from the plasmids. Replacement of the missing IL-3 and mIL-6 sequences by the oligonucleotide also serves to join the IL-3 and mIL-6 sequences together to form the chimeric IL-3/mIL-6 nucleic acid sequence in such a way that both interleukins are in frame for translation;

3) the chimeric IL-3/mIL-6 nucleic acid sequence is assembled by combining the IL-3 fragment, the mIL-6 fragment, and, optionally, the oligonucleotide into a plasmid. The plasmid contains a selectable marker, such as an antibiotic resistance gene.

4) the chimeric IL-3/mIL-6 sequence is amplified by, for example, PCR or cloning;

5) the amplified chimeric IL-3/mIL-6 sequence is inserted into an expression vector for expression of the chimeric IL-3/mIL-6 protein. Preferably, a controllable protein expression system that causes the juxtaposition of a promoter to control the amino acid coding sequence as a non-fusion process is employed. The system can utilize any of several well-known, characterized and available promoters such as *trp*, *trc*, *tic*, *tac*, *lac*, P_L, etc.

6) following expression of the chimeric IL-3/mIL-6 protein, the chimera is isolated and purified by methods known in the art.

The starting materials for construction of the present invention are nucleic acid sequences that encode native IL-3 and either native IL-6 or mIL-6. Nucleic acid sequences encoding native IL-3 and IL-6 may be isolated from a human cDNA or genomic DNA library.

The preferred method for obtaining DNA suitable as a starting material for construction of DNA encoding the chimera of the invention is to isolate DNA encoding native IL-3 and mIL-6 from an available recombinant plasmid. Recombinant plasmids that encode native full length IL-3 and mIL-6 are known. For IL-3, see, for example, PCT publication WO 88/00598, published 28 January 1988 and PCT publication 92/04455, published 19 March 1992. For mIL-6, see, for example, Skelly et al., U.S. application 07/907,710; Dagan et al., *Protein Expression and Purification* 3, 290-294 (1992); and Snouwaert, J., et al., *J. Immunol.* 146, 585-591 (1991).

If native IL-6 DNA is used as a starting material, mIL-6 is produced by mutating the native sequence. For example, muteins may be introduced into native IL-6 by site-directed mutagenesis, in order to encode amino acid residues other than cysteine at amino acid positions 45 and 51. Site-directed

mutagenesis is carried out by methods known in the art. See, for example, Zoller and Smith, Nucl. Acids Res. 10, 6487-6500 (1982); Methods in Enzymology 100, 468-500 (1983); and DNA 3, 479-488 (1984).

Recombinant plasmids that encode native IL-6 containing the four cysteine residues are known; see, for example, Clark et al., PCT application WO88/00206; Brakenhoff et al., Journal of Immunology 143, 1175-1182 (1989); Brakenhoff et al., Journal of Immunology 139, 4116-4121 (1987); Hirano et al., Proc. Natl. Acad. Sci. USA 84, 228-231 (1987). The codons for the cysteine residues at positions corresponding to positions 45 and 51 of native IL-6 are replaced by codons for other amino acids, preferably by codons for any other neutral amino acids, and more preferably by codons for serine or alanine residues.

Alternatively, plasmids containing DNA that encodes variants of native IL-6 in which all four cysteine residues have been replaced by serine residues may be obtained as described in Fowlkes et al., PCT application US89/05421. The codons for the serine residues at positions corresponding to positions 74 and 84 of native IL-6 are replaced by cysteine residues by, for example, site-directed mutagenesis. The codons for the serine residues at positions corresponding to 45 and 51 may be retained or replaced by other amino acid residues, such as by alanine, in the same way.

As an alternative, DNA encoding IL-3, IL-6, mIL-6 or the IL-3/mIL-6 chimera may be synthesized from individual nucleotides. Chemical synthesis of DNA from the four nucleotides may be accomplished in whole or in part by methods known in the art. Such methods include those described by Caruthers in Science 230, 281-285 (1985). DNA may also be synthesized by preparing

overlapping double-stranded oligonucleotides, filling in the gaps, and ligating the ends together.

Construction of the chimeric DNA sequences that encode the protein of the present invention is described below in Example 1.

The DNA obtained may be amplified by methods known in the art. One suitable method is the polymerase chain reaction (PCR) method described by Saiki et al. in Science 239, 487 (1988), Mullis et al in U.S. Patent 4,683,195 and by Sambrook, Fritsch and Maniatis (eds) in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989). It is convenient to amplify the clones in the lambda-gt10 or lambda-gt11 vectors using lambda-gt10 or lambda-gt11-specific oligomers as the amplimers (available from Clontech, Palo Alto, California).

The DNA fragments encoding the protein of the invention may be assembled in the proper order and replicated following insertion into a wide variety of host cells in a wide variety of cloning vectors. The host may be prokaryotic or eukaryotic.

Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from *E.coli*, such as colE1, pCR1, pBR322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13 fd, and other filamentous single-stranded DNA phages.

Vectors for expressing proteins in bacteria, especially *E.coli*, are also known. Such vectors include the pK233 (or any of the *tac* family of plasmids), T7, and lambda P_L. Examples of vectors that express fusion proteins are PATH

vectors described by Dieckmann and Tzagoloff in J. Biol. Chem. 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); glutathione S-transferase (pGST) - see Gene 67, 31 (1988) and Peptide Research 3, 167 (1990).

Vectors useful for cloning and expression in yeast are available. A suitable example is the 2m circle plasmid.

Suitable cloning/expression vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV) retrovirus-derived DNA sequences. Any such vectors, when coupled with vectors derived from a combination of plasmids and phage DNA, i.e. shuttle vectors, allow for the isolation and identification of protein coding sequences in prokaryotes.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, J. Mol. Appl. Genet. 1, 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159, 601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Useful expression hosts include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DHI, and *E. coli* MRCI, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

The chimeric protein of the invention may be expressed in the form of a fusion protein with an appropriate fusion partner. The fusion partner preferably facilitates purification and identification. Increased yields may be achieved when the fusion partner is expressed naturally in the host cell. Some useful fusion partners include beta-galactosidase (Gray, et al., Proc. Natl. Acad. Sci. USA 79, 6598 (1982)); trpE (Itakura et al., Science 198, 1056 (1977)); protein A (Uhlen et al., Gene 23 369 (1983)); glutathione S-transferase (Johnson, Nature

338, 585 (1989)); Van Etten et al., Cell 58, 669 (1989)); and maltose binding protein (Guan et al., Gene 67, 21-30 (1987); Maina et al., Gene 74, 36-373 (1988); Riggs, P., in Ausebel, F.M. et al (eds) Current Protocols in Molecular Biology, Greene Associates/Wiley Interscience, New York (1990)).

Such fusion proteins may be purified by affinity chromatography using reagents that bind to the fusion partner. The reagent may be a specific ligand of the fusion partner or an antibody, preferably a monoclonal antibody. For example, fusion proteins containing beta-galactosidase may be purified by affinity chromatography using an anti-beta-galactosidase antibody column (Ullman, Gene. 29, 27-31 (1984)). Similarly, fusion proteins containing maltose binding protein may be purified by affinity chromatography using a column containing cross-linked amylose; see Guan, European Patent Application 286,239.

The fusion protein may occur at the amino-terminal or the carboxy-terminal side of the cleavage site. Optionally, the DNA that encodes the fusion protein is engineered so that the fusion protein contains a cleavable site between the protein and the fusion partner. Both chemical and enzymatic cleavable sites are known in the art. Suitable examples of sites that are cleavable enzymatically include sites that are specifically recognized and cleaved by collagenase (Keil et al., FEBS Letters 56, 292-296 (1975)); enterokinase (Hopp et al., Biotechnology 6, 1204-1210 (1988)); factor Xa (Nagai et al., Methods Enzymol. 153, 461-481 (1987)); and thrombin (Eaton et al., Biochemistry 25, 505 (1986)). Collagenase cleaves between proline and X in the sequence Pro-X-Gly-Pro wherein X is a neutral amino acid. Enterkinase cleaves after lysine in the sequence Asp-Asp-Asp-Asp-Lys. Factor Xa cleaves after arginine in the sequence Ile-Glu-Gly-Arg. Thrombin cleaves between arginine and glycine in the sequence Arg-Gly-Ser-Pro.

Specific chemical cleavage agents are also known. For example, cyanogen bromide cleaves at methionine residues in proteins.

The chimeric protein is purified by methods known in the art. Such methods include affinity chromatography using specific antibodies. Alternatively, the recombinant protein may be purified using a combination of ion-exchange, size-exclusion, and hydrophobic interaction chromatography using methods known in the art. These and other suitable methods are described by Marston, "The Purification of Eukaryotic Proteins Expressed in E. coli" in DNA Cloning, D. M. Glover, Ed., Volume III, IRL Press Ltd., England, 1987.

SEQ. ID. NOS. 1-2 show the amino acid sequence of one chimeric IL-3/mIL-6 protein of the invention. This sequence shows an embodiment in which the carboxy terminal end of IL-3 is attached to the amino terminal end of mIL-6. A nucleotide sequence that expresses the chimer is also shown in SEQ. ID. NO. 1.

In addition another mutein of IL-6 which has increased activity resulting from an amino acid substitution at, or corresponding to, amino acid location 171 or 175 of IL-6 having the wild-type sequence has been described by Leebeek, F.W.G., et al., *J. Biol. Chem.* 267 (21) 14832-14838 (1992). Substitutions of these carboxy-terminal amino acids may be introduced into the mIL-6 portion of the chimer of the present invention.

The invention also includes equivalent variants of the IL-3 and mIL-6 portions of the chimeric protein described above and the nucleic acid molecules that encode such variants. Equivalent variants include proteins comprising

substitutions and additions in the amino acid and nucleotide sequences of the chimeras of the invention and the corresponding nucleic acid molecules. Variants are included in the invention as long as the resulting chimeras and nucleic acid molecules continue to satisfy the structural and functional criteria described above, i.e., retain activity at least comparable to that of native IL-3 and mIL-6 and lack cysteine residues at positions 45 and 51 of the IL-6 portion. An amino acid or nucleotide sequence that is substantially the same as another sequence, but that differs from the other sequence by means of one or more substitutions or additions is considered to be an equivalent sequence. Except for the substitutions of cysteine residues at positions corresponding to positions 45 and 51 of native, mature IL-6, preferably less than 25%, more preferably less than 10%, and most preferably less than 5% of the total number of amino acids or nucleotides in the chimeras of the invention are substituted for or added to in the equivalent sequences.

For example, it is known to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids considered normally to be equivalent are:

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);
(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V); and
(e) Phe(F) Tyr(Y) Trp(W).

Additions to the IL-3/mIL-6 muteins may be made at the C-terminal or N-terminal ends by adding the corresponding codons at the 5' or 3' ends of the nucleic acid sequences and expressing the nucleic acid molecules. Examples of internal additions to the nucleic acid molecules include the introns present in

genomic DNA. The introns are not expressed in a suitable eukaryotic host cell.

Equivalents of the nucleic acid molecules encoding the chimeric IL-3/mIL-6 protein also include silent mutations at sites that do not alter the amino acid sequence expressed. Preferably, the silent mutation results in increased expression in a particular host.

The chimera may contain the entire IL-3 and mIL-6 proteins, or a biologically active fragment of either or both whole proteins. Bioactive fragments of bioactive proteins may be identified by methods known in the art. For example, IL-6 fragments lacking amino acids 1-28 are known to be active. See, for example, Brakenhoff, J.P.J., et al., *J. Immunol.* 143, 1175-1182 (1989).

Fragments containing bioactive sequences may be selected on the basis of generally accepted criteria of potential bioactivity. Such criteria include analysis of which region(s) of a protein is required for bioactivity.

Methods for determining the biological activity of chimeric interleukin proteins are described in example 9 of PCT publication WO 92/04455, published 19 March 1992.

Nucleic Acid Molecules

The present invention includes nucleic acid molecules that encode the chimera of the present invention. Any nucleic acid sequence that encodes the amino acid sequence of SEQ. ID. NOS. 1-2 can be used to express the chimeric protein of the present invention. For example, nucleic acid sequences

that are found in nature or can be selected that will maximize expression in bacteria. The nucleic acid molecule may be DNA or RNA.

The nucleic acid molecules may be used as probes for detecting DNA encoding IL-3, IL-6, mIL-6 or chimeric IL-3/mIL-6 as explained below, or to produce a protein of the invention, as explained above.

Probes

The chimeric protein and DNA can be used to prepare probes that detect the presence of IL-3, IL-6, mIL-6 or the chimeric IL-3/mIL-6 protein or DNA in a sample. The method involves use of a labelled probe that recognizes IL-3, IL-6, mIL-6 or the chimeric IL-3/IL-6 protein or DNA present in biological samples, including, but not limited to, lymphatic fluid, synovial fluid, cerebral-spinal fluid, blood, tissue and cell samples. The probe may be an antibody raised against the chimeric IL-3/mIL-6 protein, or a fragment thereof, or an oligonucleotide that hybridizes to DNA encoding IL-3, IL-6, mIL-6 or the chimeric IL-3/mIL-6 protein. The antibody may be polyclonal or monoclonal.

Preparing Antibodies

Polyclonal antibodies are isolated from mammals that have been innoculated with the chimeric protein or a functional analog in accordance with methods known in the art. Briefly, polyclonal antibodies may be produced by injecting a host mammal, such as a rabbit, mouse, rat, or goat, with the chimeric protein or a fragment thereof. Sera from the mammal are extracted and screened to obtain polyclonal antibodies that are specific to the chimeric protein or protein fragment.

The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in Nature 256, 495-497 (1975) and by Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds, Laboratoty Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); as well as the recombinant DNA method described by Huse et al. in Science 246, 1275-1281 (1989).

The probes described above are labelled in accordance with methods known in the art. The label may be a radioactive atom, an enzyme, or a chromophoric moiety.

Methods for labelling antibodies have been described, for example, by Hunter and Greenwood in Nature 144, 945 (1962) and by David et al. in Biochemistry 13, 1014-1021 (1974). Additional methods for labelling antibodies have been described in U.S. patents 3,940,475 and 3,645,090.

Methods for labelling oligonucleotide probes have been described, for example, by Leary et al., Proc. Natl. Acad. Sci. USA (1983) 80:4045; Renz and Kurz, Nucl. Acids Res. (1984) 12:3435; Richardson and Gumport, Nucl. Acids Res. (1983) 11:6167; Smith et al., Nucl. Acids Res. (1985) 13:2399; and Meinkoth and Wahl, Anal. Biochem. (1984) 138:267.

The label may be radioactive. Some examples of useful radioactive labels include ^{32}P, ^{125}I, ^{131}I, and ^{3}H. Use of radioactive labels have been described in U.K. 2,034,323, U.S. 4,358,535, and U.S. 4,302,204.

Some examples of non-radioactive labels include enzymes, chromophors, atoms and molecules detectable by electron microscopy, and metal ions detectable by their magnetic properties.

Detecting Protein with Antibodies

The probe may be an antibody, preferably a monoclonal antibody. The antibodies may be prepared as described above.

Assays for detecting the presence of proteins with antibodies have been previously described, and follow known formats, such as standard blot and ELISA formats. These formats are normally based on incubating an antibody with a sample suspected of containing the protein and detecting the presence of a complex between the antibody and the protein. The antibody is labelled either before, during, or after the incubation step. The protein is preferably immobilized prior to detection. Immobilization may be accomplished by directly binding the protein to a solid surface, such as a microtiter well, or by binding the protein to immobilized antibodies.

In a preferred embodiment, a protein is immobilized on a solid support through an immobilized first antibody specific for the protein. The immobilized first antibody is incubated with a sample suspected of containing the protein. If present, the protein binds to the first antibody.

A second antibody, also specific for the protein, binds to the immobilized protein. The second antibody may be labelled by methods known in the art. Non-immobilized materials are washed away, and the presence of immobilized label indicates the presence of the protein. This and other immunoassays are

described by David, et al. in U.S. Patent 4,376,110 assigned to Hybritech, Inc., LaJolla, California.

Detecting Antibodies with Protein

The chimeric protein may be labelled and used as probes in standard immunoassays to detect antibodies against IL-3, IL-6, mIL-6 or chimeric IL-3/mIL-6 proteins in samples, such as in the sera or other bodily fluids of patients. In general, a protein in accordance with the invention is incubated with the sample suspected of containing antibodies to the protein. The protein is labelled either before, during, or after incubation. The detection of labelled protein bound to an antibody in the sample indicates the presence of the antibody. The antibody is preferably immobilized.

Suitable assays are known in the art, such as the standard ELISA protocol described by R.H. Kenneth, "Enzyme-Linked Antibody Assay with Cells Attached to Polyvinyl Chloride Plates" in Kenneth et al, Monoclonal Antibodies, Plenum Press, N.Y., page 376 (1981).

Oligonucleotide Probes

The probe may also be an oligonucleotide complementary to a target nucleic acid molecule. The nucleic acid molecules may be RNA or DNA.

The length of the oligonucleotide probe is not critical, as long as it is capable of hybridizing to the target molecule. The oligonucleotide should contain at least 6 nucleotides, preferably at least 10 nucleotides, and, more preferably, at least 15 nucleotides.

There is no upper limit to the length of the oligonucleotide probes. Longer probes are more difficult to prepare and require longer hybridization times. Therefore, the probe should not be longer than necessary. Normally, the oligonucleotide probe will not contain more than 50 nucleotides, preferably not more than 40 nucleotides, and, more preferably, not more than 30 nucleotides.

The chimeric IL-3/mIL-6 protein of the present invention possesses *in vitro* and *in vivo* biological activity at least comparable to that of a mixture of IL-3 and IL-6 or IL-3 and mIL-6. Accordingly, the chimeric IL-3/mIL-6 protein is useful in the *in vitro* and *in vivo* stimulation of the formation, proliferation and differentiation of a broad range of hematopoietic cells, including granulocytes, macrophages, eosinophils, mast cells, erythroid cells, B cells, T cells, megakaryocytes, and multi-potential hematopoietic progenitor cells. The stimulation of proliferation of megakaryocytes leads to the production of platelets. In addition, the mIL-6 portion of the chimeric IL-3/mIL-6 protein induces various acute phase proteins in liver cells. As a result of these biological activities, the chimeric IL-3/mIL-6 protein is useful in immunotherapeutic and anti-inflammation compositions. The chimera may also be used for the treatment of patients suffering from thrombocytopenia and patients undergoing chemotherapy or bone marrow transfers.

EXAMPLES

Example 1.

A. Construction of the Chimeric IL-3/mIL-6 Nucleic Acid Sequence.

The starting material for the construction of the chimeric IL-3/mIL-6 nucleic acid sequence is a plasmid, designated p570 (ATCC 69242). The p570

plasmid contains the cloned mature human IL-3 gene. An analogous plasmid containing sequences that encode mature human IL-3 can be obtained from R&D Systems Inc., Minneapolis, Mn., catalog No. BBG 14. Mature human IL-3 contains 133 amino acids. (See line A in Figure 1 and SEQ. ID. NO. 3-4)

The p570 plasmid is digested with the restriction endonucleases *Nco*I and *Dde*I. (New England Bio Labs, Beverly, Ma.) Digestion of the plasmid with these enzymes liberates a 0.375 kbp fragment (Line B in Figure 1) which encodes the natural amino terminus of human IL-3 and extends toward the carboxy terminus of the protein to the codon encoding alanine at amino acid position number 121. (See SEQ. ID. NO. 5)

The mIL-6 nucleic acid sequences are obtained from a plasmid designated pKK233-2 IL-6 SSCC. (See SEQ. ID. NO. 6-7 for the portion of the plasmid the encodes the sequence of mIL-6) Construction of the plasmid is described by Skelly et al., in example 5 of co-pending U.S. application 07/907,710, which is incorporated herein by reference and in Dagan et al., *Protein Expression and Purification* 3, 290-294 (1992). The pKK233-2 IL-6 SSCC plasmid contains a 0.6 kbp *Nco*I/*Hind*III restriction fragment that encodes mature mIL-6. The *Nco*I restriction site of this plasmid places an ATG codon immediately upstream of the initial mIL-6 amino acid residue, alanine. The *Nco*I site is followed 12 bp downstream by a unique *EcoR*II recognition sequence. As shown in Figure 1, when pKK233-2 IL-6 SSCC is digested with *EcoR*II and *Hind*III restriction enzymes (New England Bio Labs, Beverly, Ma.), a 0.59 kbp fragment is generated. (See line E and SEQ. ID. NO. 8) This fragment encodes the complete mIL-6 product minus the alanine-proline-valine-proline amino terminal residues and is followed by a *Kpn*I restriction site and three random in-frame stop codons.

Since the nucleic acid sequences encoding the last eleven amino acids from IL-3 and the first four amino acids from mIL-6 are lost as a result of the restriction endonuclease excision of the genes from their respective plasmids, an oligonucleotide pair (lines D and D' in Figure 1) encoding the lost amino acids is used to replace the lost nucleic acid sequences. In addition to replacing the lost nucleic acid sequences, the oligonucleotide pair (lines D and D' in Figure 1) join the IL-3 fragment (line B in Figure 1) to the mIL-6 fragment (line E in Figure 1) to form a chimeric IL-3/mIL-6 cassette with *Nco*I and *Hind*III termini. (See SEQ. ID. NO. 9) Synthesis of the oligonucleotides is described below in Section B. (See SEQ. ID. NOS. 10-11)

The chimeric IL-3/mIL-6 cassette is assembled by simultaneously combining the IL-3 fragment (component 1; line B in Figure 1), the mIL-6 fragment (component 2; line E in Figure 1) and the oligonucleotide pair (component 3; lines D and D' in Figure 1) with a plasmid (component 4) that has been pre-digested and purified by standard methods to remove a *Nco*I/*Hind*III restriction fragment from its sequence. The plasmid used in this example is designated pKK233-2 (Pharmacia LKB, Piscataway, N.J.). Once assembled, the chimeric IL-3/mIL-6 cassette, which has *Nco*I and *Hind*III termini, replaces the original *Nco*I/*Hind*III restriction fragment in the plasmid. The pKK233-2 plasmid contains an ampicillin resistance gene that is rendered functional if the four components of the reaction correctly assemble themselves to form the chimeric IL-3/mIL-6-pKK233-2 plasmid. The plasmid is transfected into *E.coli*. *E.coli* containing the chimeric IL-3/mIL-6 nucleic acid in the plasmid are selected for by growing the bacteria on agar containing ampicillin. Once selected, the IL-3/mIL-6-pKK233-2 plasmid is amplified to desired levels by growing the bacteria in a standard culture. (See Sambrook, Fritsch and Maniatis (eds) in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989)) The ampicillin-resistant clone is verified as

having the IL-3/mIL-6 gene by restriction enzyme analysis, sequencing data (Sanger, et al., 1977 Proc. Nat. Acad. of Sci., 74:5463) and expression of the IL-3/mIL-6 protein.

Expression of the IL-3/mIL-6 chimeric protein in *E.coli* is accomplished by inserting the chimeric IL-3/mIL-6 nucleic acid sequence into an expression vector. The expression vector pSE420 (*In Vitrogen*, San Diego, Ca.) contains the $lacI^q$ gene which allows for regulated expression in *E.coli* HB101. Transcriptional control is via the *trc* promoter and utilizes the highly efficient translation re-initiation characteristic of mini-cistron systems. The incorporation of upstream anti-termination and g10 ribosome binding sequences ensures high level translation of inserts cloned into its polylinker. Digestion of pSE420 with *Nco*I and *Kpn*I (New England Bio Labs, Beverly, Ma.) allows subsequent mobilization of the IL-3/mIL-6 chimera into this protein expression system by *NcoI/KpnI* digestion of IL-3/mIL-6-pKK233-2 plasmid. (See SEQ. ID. NO. 12 for the sequence of the *NcoI/KpnI* fragment) The resulting product is illustrated in Figures 2 and 3.

B. Synthesis of Oligonucleotides.

Oligonucleotide chains are specifically synthesized on a Model 392 Applied Biosystems apparatus utilizing beta-cyanoethyl phosphoramidites as substrate. Synthesized nucleotide oligomers are deprotected and cleaved from resin supports using standard procedures as recommended by the manufacturer. One may utilize any of a variety of oligonucleotide purification cartridges or proceed with HPLC purification and isolation.

C. Expression of Chimeric IL-3/mIL-6 Protein in *E.coli*.

Expression of the chimeric IL-3/mIL-6 protein is induced in high yield with isopropyl-beta-D-thiogalactopyranoside in E. coli strains HB101.

D. Purification of Chimeric IL-3/mIL-6 Protein.

Following expression of chimeric IL-3/mIL-6 protein in *E.coli*, the bacteria are harvested by centrifugation at 4°C and washed once in cold PBS. Bacterial pellets are suspended in 5ml/gm of cold 50mM Tris-HCl (pH 8.0), 100 mM NaCl, 1mM EDTA. Protease inhibitors PMSF (0.5mM), leupeptin (5mg/ml), aprotinin (5mg/ml) are included. Lysozyme, 50mg, is added and the suspension held on ice for 30 minutes. An equal volume of lysis buffer (50mm Tris-HCl, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate) is added and the mixture gently rocked at room temperature for 30 minutes. $MgSO_4$ is added to a final concentration of 50mM followed by 25mg DNAasel (New England Bio Labs, Beverly, Ma.). The mixture is incubated at room temperature until viscosity is minimal. This solution is then centrifuged at 10k rpm in a Beckman JS 13.1 swing-bucket rotor at 4°C. The pellet is washed once in Tris-HCl (pH 8.0), 100 mM NaCl and resuspended in this solution for protein determination by BioRad (Richmond, Ca.) assay.

Large Scale Chimera Purification

Frozen E. coli cell pellets (10g) are suspended in 50mM Tris-HCl pH 8.5, 5mM EDTA, 1mM AEBSF (buffer A). Lysozyme is added to a final concentration of 300mg/ml and the lysate is incubated on ice for 30 minutes. The lysate is homogenized on ice and then centrifuged at 10,000Xg for 30 minutes. The resulting pellet is washed 2X by centrifugation with buffer A containing 0.5% Triton X-100 and the supernatants discarded. The final pellet

containing chimeric IL-3/mIL-6 inclusion bodies is resuspended in 50mM Tris-HCl pH 8.5, 6M guanidine-HCl, 1mM EDTA, 5mM DTT, 0.1mM AEBSF and incubated at room temperature for 2 hours. The extract is then clarified by centrifugation at 15,000Xg for 1hr.

The solubilized IL-3/mIL-6 is refolded by diluting the extract ten fold with 50mM Tris-HCl pH 8.5, 100mM NaCl, 1mM EDTA, 0.1mM AEBSF and incubating for 36hrs at 4°C. The protein concentration during refolding is < 0.2mg/ml. Insoluble material is removed by centrifugation and the supernatant dialyzed against 20mM Tris-HCl pH 8.5, 1mM EDTA, 0.1mM DTT.

Dialyzed IL-3/mIL-6 is applied to a Q-Sepharose HP (Pharmacia LKB, Piscataway, N.J.) anion exchange column (1.6 X 10cm) equilibrated in 20mM Tris-HCl pH 8.5 and eluted with a linear gradient of 500mM NaCl. Fractions containing the chimeric IL-3/mIL-6 are identified by ELISA, pooled and loaded onto a C4 reverse-phase column (Vydac C4, 4.6mm X 250mm) equilibrated in 100mM ammonium acetate (pH 6.0):isopropanol(85:15). The IL-3/mIL-6 is eluted with a linear gradient of 100mM ammonium acetate (pH 6.0):isopropanol (18:82) over 80 minutes at a flow rate of 0.7ml/min. Fractions containing purified IL-3/mIL-6 are pooled and stored at -70°C.

Final purity of the chimeric IL-3/mIL-6 is >90% as determined by silver stained SDS-PAGE gels. The final yield of purified IL-3/IL-6 from 10 grams of cell paste (wet weight) is ~350mg.

SUPPLEMENTAL ENABLEMENT

The invention as claimed is enabled in accordance with the specification and readily available references and starting materials.

Nevertheless, on February 8, 1993, Applicants have deposited with the American Type Culture Collection, Rockville, Md., USA (ATCC) the bacterial plasmid listed below: These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

NAME	Accession No.
p570	69242

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ImClone Systems Incorporated

(ii) TITLE OF INVENTION: Chimeric Interleukin-3/Interleukin-6 Lymphokine

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ImClone Systems Incorporated
(B) STREET: 180 Varick Street
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10014

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Feit, Irving N.
(B) REGISTRATION NUMBER: 28,601
(C) REFERENCE/DOCKET NUMBER: TAC-4-T

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-645-1405
(B) TELEFAX: 212-645-2054

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 968 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..962

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 3..959

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CC ATG GCT CCG ATG ACC CAG ACC ACC TCC CTG AAA ACC TCC TGG
GTT      47
  Met Ala Pro Met Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val
   1           5              10              15

AAC TGT TCG AAC ATG ATC GAC GAA ATC ATC ACC CAC CTG AAA CAG
CCG      95
Asn Cys Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro
            20              25              30

CCG CTG CCG CTT CTA GAC TTC AAC AAC CTG AAC GGT GAA GAC CAG
GAC      143
Pro Leu Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp
         35              40              45

ATC CTG ATG GAA AAC AAC CTG CGT CGA CCG AAC CTG GAA GCA TTC
AAC      191
Ile Leu Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn
      50              55              60

CGT GCT GTT AAA AGC TTG CAG AAC GCT TCC GCT ATC GAA TCC ATC
CTG      239
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Arg Ala Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu
65 70 75

AAA AAC CTG CTG CCG TGC CTG CCG CTG GCT ACC GCG GCT CCG ACC CGT 287
Lys Asn Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg
80 85 90 95

CAC CCG ATC CAC ATC AAA GAC GGT GAC TGG AAC GAA TTT CGT CGT AAA 335
His Pro Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys
100 105 110

CTG ACC TTC TAC CTG AAA ACC CTC GAG AAC GCT CAG GCT CAG CAG ACC 383
Leu Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr
115 120 125

ACC CTG TCC CTG GCT ATC TTC GCT CCG GTT CCG CCA GGA GAA GAT TCC 431
Thr Leu Ser Leu Ala Ile Phe Ala Pro Val Pro Pro Gly Glu Asp Ser
130 135 140

AAA GAT GTA GCC GCC CCA CAC AGA CAG CCG CTC ACC TCT TCA GAA CGA 479
Lys Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg
145 150 155

ATC GAT AAA CAA ATT CGG TAC ATC CTC GAC GGG ATA TCA GCG CTG AGA 527
Ile Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg
160 165 170 175

AAA GAG ACC AGC AAC AAG AGT AAC ATG AGC GAA AGC AGT AAA GAA GCA 575
Lys Glu Thr Ser Asn Lys Ser Asn Met Ser Glu Ser Ser Lys Glu Ala
180 185 190

CTG GCA GAA AAC AAC CTG AAC CTT CCG AAG ATG GCT GAA AAA GAT GGA 623
Leu Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys Asp Gly
195 200 205

TGT TTT CAA TCT GGA TTC AAT GAG GAA ACT TGT CTG GTG AAA ATC
ATC 671
Cys Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile
210 215 220

ACA GGC CTT TTG GAA TTT GAG GTA TAC CTA GAG TAC CTC CAG AAC
AGA 719
Thr Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg
225 230 235

TTT GAG AGT AGT GAG GAA CAA GCG AGA GCT GTC CAG ATG TCG ACC
AAA 767
Phe Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys
240 245 250 255

GTC CTG ATC CAG TTT CTG CAG AAA AAG GCA AAA AAT CTA GAT GCA
ATA 815
Val Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile
260 265 270

ACC ACC CCG GAT CCA ACC ACA AAT GCG AGC CTG CTG ACG AAG
CTG CAG 863
Thr Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln
275 280 285

GCA CAG AAC CAG TGG CTG CAG GAC ATG ACA ACT CAT CTC ATT CTG
AGA 911
Ala Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu Arg
290 295 300

TCT TTC AAA GAA TTC CTG CAG TCC TCC CTG CGT GCT CTG CGT CAG
ATG 959
Ser Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met
305 310 315

TAATGATAG 968

320

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 319 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Met Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val Asn
1 5 10 15

Cys Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro Pro
20 25 30

Leu Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp Ile
35 40 45

Leu Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn Arg
50 55 60

Ala Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu Lys
65 70 75 80

Asn Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg His
85 90 95

Pro Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys Leu
100 105 110

Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr Thr
115 120 125

Leu Ser Leu Ala Ile Phe Ala Pro Val Pro Pro Gly Glu Asp Ser Lys
130 135 140

Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile
145 150 155 160

Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys
165 170 175

Glu Thr Ser Asn Lys Ser Asn Met Ser Glu Ser Ser Lys Glu Ala Leu
180 185 190

Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys Asp Gly Cys

195 200 205

Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile Thr
210 215 220

Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe
225 230 235 240

Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys Val
245 250 255

Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr
260 265 270

Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala
275 280 285

Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu Arg Ser
290 295 300

Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met
305 310 315

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 404 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 3..404

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..404

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CC ATG GCT CCG ATG ACC CAG ACC ACC TCC CTG AAA ACC TCC TGG GTT 47
Met Ala Pro Met Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val
1 5 10 15

AAC TGT TCG AAC ATG ATC GAC GAA ATC ATC ACC CAC CTG AAA CAG CCG 95
Asn Cys Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro
20 25 30

CCG CTG CCG CTT CTA GAC TTC AAC AAC CTG AAC GGT GAA GAC CAG GAC 143
Pro Leu Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp
35 40 45

ATC CTG ATG GAA AAC AAC CTG CGT CGA CCG AAC CTG GAA GCA TTC AAC 191
Ile Leu Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn
50 55 60

CGT GCT GTT AAA AGC TTG CAG AAC GCT TCC GCT ATC GAA TCC ATC CTG 239
Arg Ala Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu
65 70 75

AAA AAC CTG CTG CCG TGC CTG CCG CTG GCT ACC GCG GCT CCG ACC CGT 287
Lys Asn Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg
80 85 90 95

CAC CCG ATC CAC ATC AAA GAC GGT GAC TGG AAC GAA TTT CGT CGT AAA 335
His Pro Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys
100 105 110

CTG ACC TTC TAC CTG AAA ACC CTC GAG AAC GCT CAG GCT CAG CAG
ACC 383
Leu Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr
115 120 125

ACC CTG TCC CTG GCT ATC TTC 404
Thr Leu Ser Leu Ala Ile Phe
130

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 134 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Pro Met Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val Asn
1 5 10 15

Cys Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro Pro
20 25 30

Leu Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp Ile
35 40 45

Leu Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn Arg
50 55 60

Ala Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu Lys
65 70 75 80

Asn Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg His
85 90 95

Pro Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys Leu
100 105 110

Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr Thr
115 120 125

Leu Ser Leu Ala Ile Phe
130

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 378 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCATGGCTCC GATGACCCAG ACCACCTCCC TTTTTTGAAA
ACCTCCTGGG TTAACTGTTC 60

GAACATGATC GACGAAATCA TCACCCACCT GAAACAGCCG
CCGCTGCCGC TTCTAGACTT 120

CAACAACCTG AACGGTGAAG ACCAGGACAT CCTGATGGAA
AACAACCTGC GTCGACCGAA 180

CCTGGAAGCA TTCAACCGTG CTGTTAAAAG CTTGCAGAAC
GCTTCCGCTA TCGAATCCAT 240

CCTGAAAAAC CTGCTGCCGT GCCTGCCGCT GGCTACCGCG
GCTCCGACCC GTCACCCGAT 300

CCACATCAAA GACGGTGACT GGAACGAATT TCGTCGTAAA
CTGACCTTCT ACCTGAAAAC 360

CCTCGAGAAC GCTCAGGC 378

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 564 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTCCGGTTC CGCCAGGAGA AGATTCCAAA GATGTAGCCG
CCCCACACAG ACAGCCGCTC 60

ACCTCTTCAG AACGAATCGA TAAACAAATT CGGTACATCC
TCGACGGGAT ATCAGCGCTG 120

AGAAAAGAGA CCAGCAACAA GAGTAACATG AGCGAAAGCA
GTAAAGAAGC ACTGGCAGAA 180

AACAACCTGA ACCTTCCGAA GATGGCTGAA AAAGATGGAT GTTTTCAATC
TGGATTCAAT 240

GAGGAAACTT GTCTGGTGAA AATCATCACA GGCCTTTTGG
AATTTGAGGT ATACCTAGAG 300

TACCTCCAGA ACAGATTTGA GAGTAGTGAG GAACAAGCGA
GAGCTGTCCA GATGTCGACC 360

AAAGTCCTGA TCCAGTTTCT GCAGAAAAAG GCAAAAAATC TAGATGCAAT
AACCACCCCG 420

GATCCAACCA CAAATGCGAG CCTGCTGACG AAGCTGCAGG
CACAGAACCA GTGGCTGCAG 480

GACATGACAA CTCATCTCAT TCTGAGATCT TTCAAAGAAT TCCTGCAGTC
CTCCCTGCGT 540

GCTCTGCGTC AGATGTAATG ATAG 564

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 585 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCAGGAGAAG ATTCCAAAGA TGTAGCCGCC CCACACAGAC
AGCCGCTCAC CTCTTCAGAA 60

CGAATCGATA AACAAATTCG GTACATCCTC GACGGGATAT
CAGCGCTGAG AAAAGAGACC 120

AGCAACAAGA GTAACATGAG CGAAAGCAGT AAAGAAGCAC
TGGCAGAAAA CAACCTGAAC 180

CTTCCGAAGA TGGCTGAAAA AGATGGATGT TTTCAATCTG GATTCAATGA
GGAAACTTGT 240

CTGGTGAAAA TCATCACAGG CCTTTTGGAA TTTGAGGTAT ACCTAGAGTA
CCTCCAGAAC 300

AGATTTGAGA GTAGTGAGGA ACAAGCGAGA GCTGTCCAGA
TGTCGACCAA AGTCCTGATC 360

CAGTTTCTGC AGAAAAAGGC AAAAAATCTA GATGCAATAA
CCACCCCGGA TCCAACCACA 420

AATGCGAGCC TGCTGACGAA GCTGCAGGCA CAGAACCAGT
GGCTGCAGGA CATGACAACT 480

CATCTCATTC TGAGATCTTT CAAAGAATTC CTGCAGTCCT CCCTGCGTGC
TCTGCGTCAG 540

ATGTAATGAT AGGTACCCGA GCTCGAATTC GTCGACCTGC AGCCA
585

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1006 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCATGGCTCC GATGACCCAG ACCACCTCCC TTTTTTGAAA
ACCTCCTGGG TTAACTGTTC 60

GAACATGATC GACGAAATCA TCACCCACCT GAAACAGCCG
CCGCTGCCGC TTCTAGACTT 120

CAACAACCTG AACGGTGAAG ACCAGGACAT CCTGATGGAA
AACAACCTGC GTCGACCGAA 180

CCTGGAAGCA TTCAACCGTG CTGTTAAAAG CTTGCAGAAC
GCTTCCGCTA TCGAATCCAT 240

CCTGAAAAAC CTGCTGCCGT GCCTGCCGCT GGCTACCGCG
GCTCCGACCC GTCACCCGAT 300

CCACATCAAA GACGGTGACT GGAACGAATT TCGTCGTAAA
CTGACCTTCT ACCTGAAAAC 360

CCTCGAGAAC GCTCAGGCTC AGCAGACCAC CCTGTCCCTG
GCTATCTTCG CTCCGGTTCC 420

GCCAGGAGAA GATTCCAAAG ATGTAGCCGC CCCACACAGA
CAGCCGCTCA CCTCTTCAGA 480

ACGAATCGAT AAACAAATTC GGTACATCCT CGACGGGATA
TCAGCGCTGA GAAAAGAGAC 540

CAGCAACAAG AGTAACATGA GCGAAAGCAG TAAAGAAGCA
CTGGCAGAAA ACAACCTGAA 600

CCTTCCGAAG ATGGCTGAAA AAGATGGATG TTTTCAATCT GGATTCAATG
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TCTGGTGAAA ATCATCACAG GCCTTTTGGA ATTTGAGGTA TACCTAGAGT
ACCTCCAGAA 720

CAGATTTGAG AGTAGTGAGG AACAAGCGAG AGCTGTCCAG
ATGTCGACCA AAGTCCTGAT 780

CCAGTTTCTG CAGAAAAAGG CAAAAAATCT AGATGCAATA
ACCACCCCGG ATCCAACCAC 840

AAATGCGAGC CTGCTGACGA AGCTGCAGGC ACAGAACCAG
TGGCTGCAGG ACATGACAAC 900

TCATCTCATT CTGAGATCTT TCAAAGAATT CCTGCAGTCC TCCCTGCGTG
CTCTGCGTCA 960

GATGTAATGA TAGGTACCCG AGCTCGAATT CGTCGACCTG CAGCCA
1006

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCAGCAGACC ACCCTGTCCC TGGCTATCTT C 31

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGATAGCC AGGGACAGGG TGGTCTGCTG A 31

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 977 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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AACAACCTGC GTCGACCGAA 180

CCTGGAAGCA TTCAACCGTG CTGTTAAAAG CTTGCAGAAC
GCTTCCGCTA TCGAATCCAT 240

CCTGAAAAAC CTGCTGCCGT GCCTGCCGCT GGCTACCGCG
GCTCCGACCC GTCACCCGAT 300

CCACATCAAA GACGGTGACT GGAACGAATT TCGTCGTAAA
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CCTCGAGAAC GCTCAGGCTC AGCAGACCAC CCTGTCCCTG
GCTATCTTCG CTCCGGTTCC 420

GCCAGGAGAA GATTCCAAAG ATGTAGCCGC CCCACACAGA
CAGCCGCTCA CCTCTTCAGA 480

ACGAATCGAT AAACAAATTC GGTACATCCT CGACGGGATA
TCAGCGCTGA GAAAAGAGAC 540

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CTGGCAGAAA ACAACCTGAA 600

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ACCTCCAGAA 720

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CCAGTTTCTG CAGAAAAAGG CAAAAAATCT AGATGCAATA
ACCACCCCGG ATCCAACCAC 840

AAATGCGAGC CTGCTGACGA AGCTGCAGGC ACAGAACCAG
TGGCTGCAGG ACATGACAAC 900

TCATCTCATT CTGAGATCTT TCAAAGAATT CCTGCAGTCC TCCCTGCGTG
CTCTGCGTCA 960

GATGTAATGA TAGGTAC 977

C'_AIMS

What is claimed is:

1. A chimeric protein comprising:

an amino portion having the amino acid sequence of interleukin-3 and a carboxy portion having the amino acid sequence of mutein interleukin-6.

2. The chimeric protein of claim 1, wherein the biological activity of the interleukin-3 portion of the chimeric protein is at least comparable to the biological activity of native interleukin-3.

3. The chimeric protein of claim 1, wherein the biological activity of the mutein interleukin-6 portion of the chimeric protein is at least comparable to the biological activity of mutein interleukin-6.

4. The chimeric protein of claim 1, wherein the biological activity of the interleukin-3 portion of the chimeric protein is at least comparable to the biological activity of native interleukin-3 and the biological activity of the mutein interleukin-6 portion of the chimeric protein is at least comparable to the biological activity of mutein interleukin-6.

5. The chimeric protein of claim 1, wherein the interleukin-3 portion is of human origin.

6. The chimeric protein of claim 1, wherein the mutein interleukin-6 portion is derived from native IL-6 of human origin.

7. The chimeric protein of claim 1, wherein the interleukin-3 portion is of human origin and the mutein interleukin-6 portion is derived from native IL-6 of human origin.

8. A chimeric protein according to the claim 1 wherein the amino acid sequence is:

Met Ala Pro Met Thr Gln Thr Thr Ser Leu Lys Thr Ser Trp Val Asn
1 5 10 15
Cys Ser Asn Met Ile Asp Glu Ile Ile Thr His Leu Lys Gln Pro Pro
20 25 30
Leu Pro Leu Leu Asp Phe Asn Asn Leu Asn Gly Glu Asp Gln Asp Ile
35 40 45
Leu Met Glu Asn Asn Leu Arg Arg Pro Asn Leu Glu Ala Phe Asn
Arg 50 55 60
Ala Val Lys Ser Leu Gln Asn Ala Ser Ala Ile Glu Ser Ile Leu Lys 65
70 75 80
Asn Leu Leu Pro Cys Leu Pro Leu Ala Thr Ala Ala Pro Thr Arg His
85 90 95
Pro Ile His Ile Lys Asp Gly Asp Trp Asn Glu Phe Arg Arg Lys Leu
100 105 110
Thr Phe Tyr Leu Lys Thr Leu Glu Asn Ala Gln Ala Gln Gln Thr Thr
115 120 125
Leu Ser Leu Ala Ile Phe Ala Pro Val Pro Pro Gly Glu Asp Ser Lys
130 135 140
Asp Val Ala Ala Pro His Arg Gln Pro Leu Thr Ser Ser Glu Arg Ile
145 150 155 160
Asp Lys Gln Ile Arg Tyr Ile Leu Asp Gly Ile Ser Ala Leu Arg Lys
165 170 175
Glu Thr Ser Asn Lys Ser Asn Met Ser Glu Ser Ser Lys Glu Ala Leu
180 185 190
Ala Glu Asn Asn Leu Asn Leu Pro Lys Met Ala Glu Lys Asp Gly Cys
195 200 205
Phe Gln Ser Gly Phe Asn Glu Glu Thr Cys Leu Val Lys Ile Ile Thr
210 215 220
Gly Leu Leu Glu Phe Glu Val Tyr Leu Glu Tyr Leu Gln Asn Arg Phe
225 230 235 240

Glu Ser Ser Glu Glu Gln Ala Arg Ala Val Gln Met Ser Thr Lys Val
245 250 255
Leu Ile Gln Phe Leu Gln Lys Lys Ala Lys Asn Leu Asp Ala Ile Thr
260 265 270
Thr Pro Asp Pro Thr Thr Asn Ala Ser Leu Leu Thr Lys Leu Gln Ala
275 280 285
Gln Asn Gln Trp Leu Gln Asp Met Thr Thr His Leu Ile Leu Arg Ser
290 295 300
Phe Lys Glu Phe Leu Gln Ser Ser Leu Arg Ala Leu Arg Gln Met
305 310 315

9. A nucleic acid molecule that encodes a chimeric protein wherein the chimeric protein comprises an amino portion having the amino acid sequence of interleukin-3 and a carboxy portion having the amino acid sequence of mutein interleukin-6.

10. The nucleic acid molecule of claim 9, wherein the biological activity of the interleukin-3 portion of the chimeric protein is at least comparable to the biological activity of native interleukin-3.

11. The nucleic acid molecule of claim 9, wherein the biological activity of the mutein interleukin-6 portion of the chimeric protein is at least comparable to the biological activity of mutein interleukin-6.

12. The nucleic acid molecule of claim 9, wherein the biological activity of the interleukin-3 portion of the chimeric protein is at least comparable to the biological activity of native interleukin-3 and the biological activity of the mutein

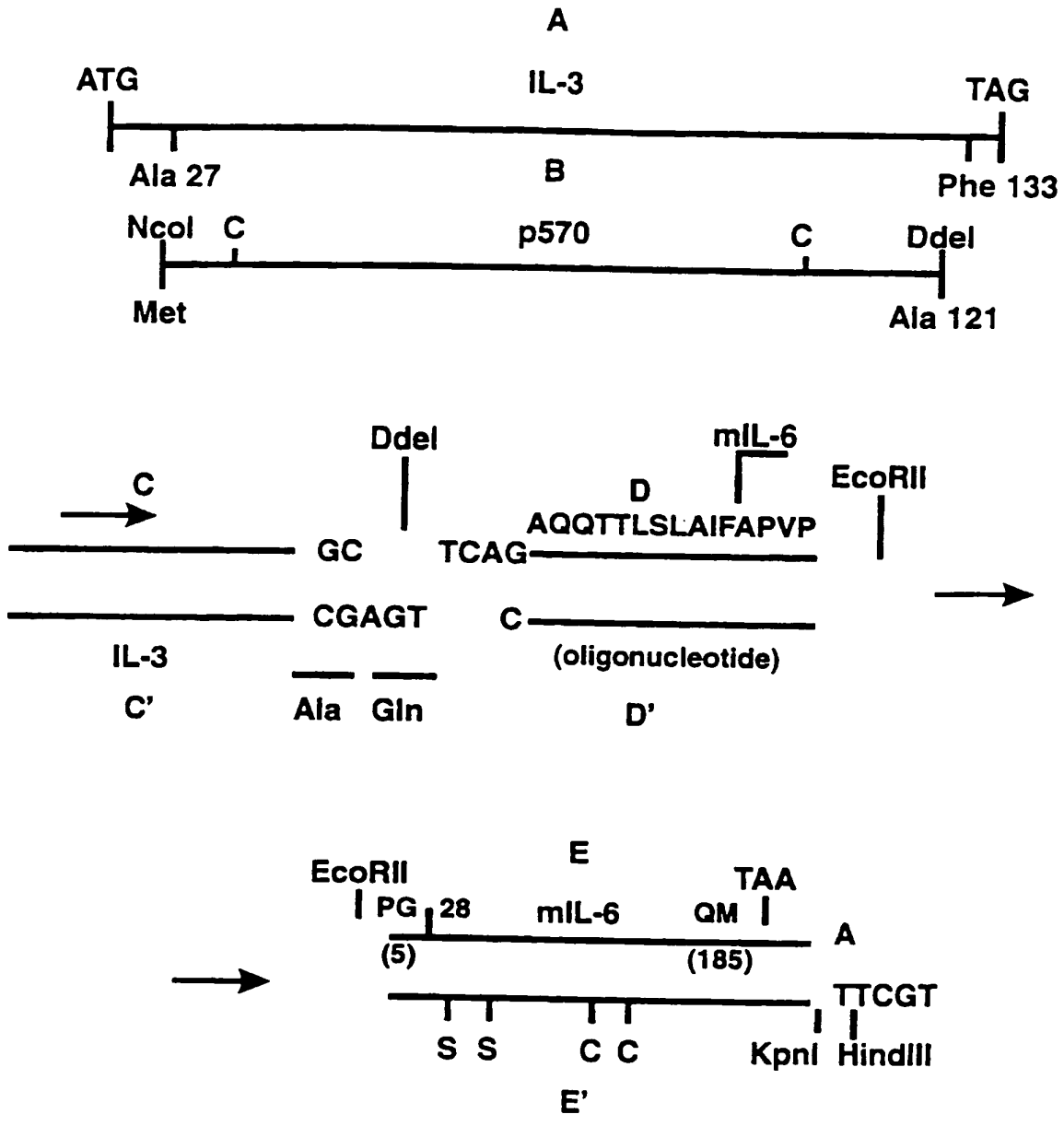
interleukin-6 portion of the chimeric protein is at least comparable to the biological activity of mutein interleukin-6.

13. The nucleic acid molecule of claim 9, wherein the interleukin-3 portion is of human origin.

14. The nucleic acid molecule of claim 9, wherein the mutein interleukin-6 portion is derived from native IL-6 of human origin.

15. The nucleic acid molecule of claim 9, wherein the interleukin-3 portion and the mutein interleukin-6 portion is derived from native IL-6 of human origin.

Figure 1


A
ATG
IL-3
TAG
Ala 27
B
Phe 133
NcoI C
p570
C
DdeI
Met
Ala 121
DdeI
mIL-6
C
D
EcoRII
AQQTTLSLAIFAPVP
GC
TCAG
CGAGT
C
IL-3
(oligonucleotide)
C'
Ala Gln
D'
E
EcoRII
TAA
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QM
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(5)
(185)
TTCGT
S S
C C
KpnI HindIII
E'

Figure 2

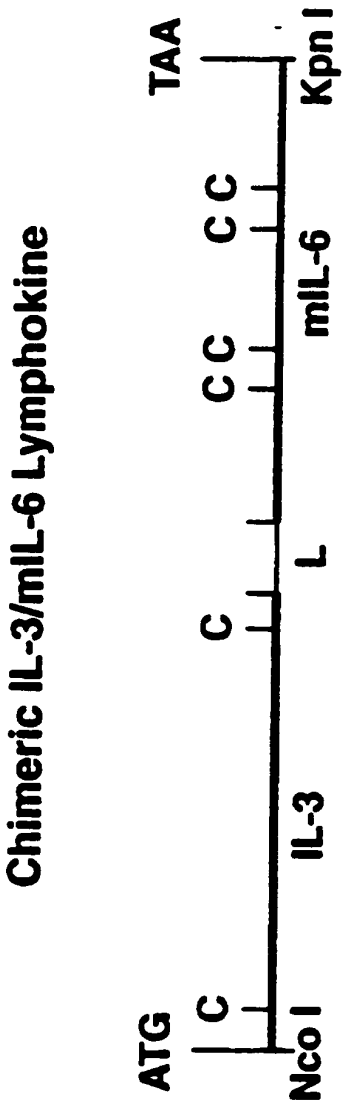
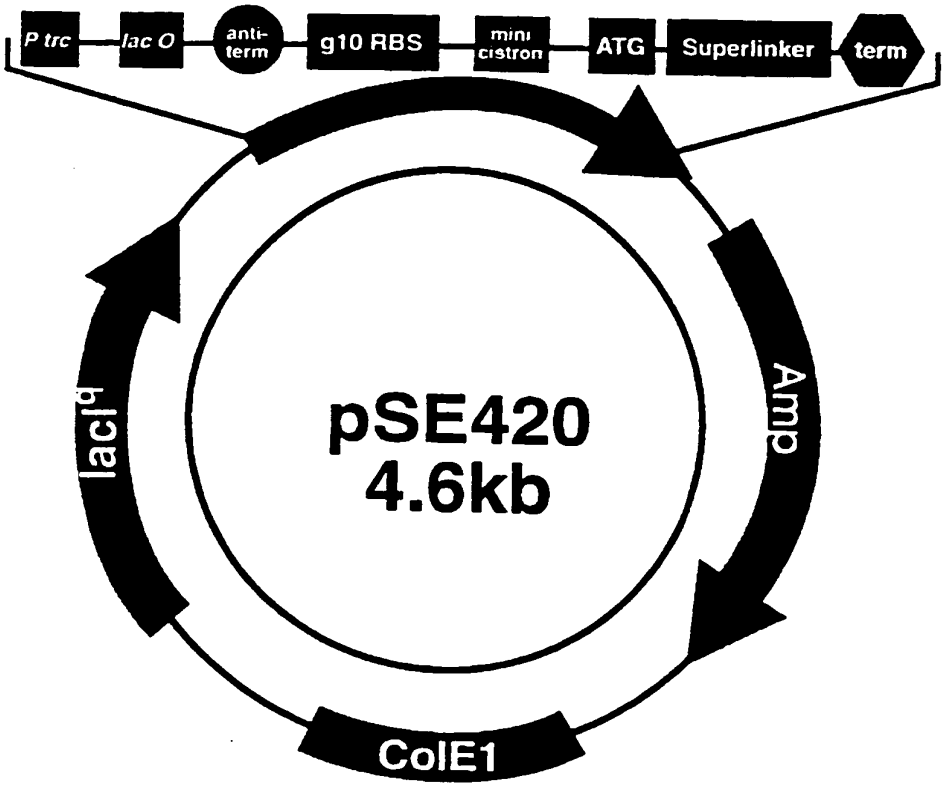

Chimeric IL-3/mIL-6 Lymphokine
ATG
C
Nco I
IL-3
C
L
C C
mIL-6
C C
TAA
Kpn I

Figure 3


P trc
lac O
anti-term
g10 RBS
mini cistron
ATG
Superlinker
term
pSE420
4.6kb
lacIq
Amp
ColE1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04208

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/00; C07H 15/12
US CL : 530/351, 402; 930/141, 142
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/351, 402; 930/141, 142

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GeneSeq, EMBL, GenBank, APS, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 5,114,711 (BELL ET AL.) 19 May 1992, see abstract.	1-15
Y	WO, A, 92/06116 (ROSEN) 16 April 1992, see the abstract, pages 6-8, and the claims.	1-15
Y	WO, A, 92/04455 (SCHENDEL) 19 March 1992, see the claims, pages 1-3, and example 7.	1-15
Y	Journal of Biological Chemistry, Volume 266, Number 34, issued 05 December 1991, Snouwaert et al., "Role of Disulfide Bonds in Biologic Activity of Human Interleukin-6", pages 23097-23102, see entire document.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 AUGUST 1994	SEP 20 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SHELLY GUEST CERMAK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04208

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Experimental Medicine, Volume 170, issued August 1989, Bergui et al., "Interleukin 3 and Interleukin 6 Synergistically Promote the Proliferation and Differentiation of Malignant Plasma Cell Precursors in Multiple Myeloma", pages 613-618, see entire document.	1-15